Method for obtaining olive leaf extracts and applications thereof

WO 2001047537 A1

ABSTRACT

The invention concerns a method for obtaining olive leaf extracts by performing extraction of olive leaves dried at a temperature of less than 35 °C with alkanols at low temperature and purifying the extract. The invention also relates to new pharmacological applications of olive leaf extracts as potentiator of cell immunity and delayed hypersensitivity in healthy humans by activating and proliferating T lymphocytes, natural killer cells, monocytes, granulocytes and pro-inflammatory cytokines.

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Inventors	Almagro Eliseo Quintanilla, 5 More »
Applicant	Asac Compania De Biotecnologia, 6 More »
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DESCRIPTION translated from Spanish (OCR text may contain errors)

TITLE OF INVENTION

Procedure for obtaining Olea europaea extracts, and applications thereof.

TECHNICAL FIELD OF THE INVENTION

The present invention describes a method for obtaining vegetable extracts from Olea europaea stabilized by extracting dried leaves less than 35 ° C, purification of the extract and evaporation of the extract. The obtained extract containing a high content and high throughput Oleuropein on the starting material.

The present invention describes new therapeutic applications of Olea europaea extracts as immunological agent, activating and proliferating T-lymphocytes, Natural Killer (NK) cells, monocytes and granulocytes in healthy humans as well as by increased proinflammatory cytokines.

STATE OF THE ART

The Oleuropein is a bitter glycoside found in the fruits, roots, barks and especially the leaves of Olea europaea, being the active component of Olea europaea extracts, along with the flavonoids in the extract.

Methods for preparing plant extracts from the leaves of Olea europaea pharmacological activity have been described in U.S. 5714150 and WO9614064.

US5714150 patent describes a process for extraction of the leaves of Olea europaea by maceration using as extractant a mixture of alcohol / water in a ratio of approximately 75% -25% at a temperature between 20-88 ° C, obtaining an extract with a Oleuropein content of 35%.

WO9614064 patent application describes obtaining Olea europaea extracts with pharmacological activity as an extractant using water and / or solutions hidroalcóholicas in a broad temperature range of 20-100 ° C based on the dried leaves.

However, no document comprised in the state of the art discloses any purification proceeds to extract or leaf drying at low temperature.

The plant extracts of Olea europaea and Oleuropein showed pharmacological activity, especially as antiviral, according to the documents that comprise the

CLAIMS translated from Spanish (OCR text may contain errors)

CLAIMS

1. Procedure for obtaining Olea europaea extracts with a yield higher than 25% comprising the steps of extraction, purification and evaporation of the solvent characterized in that the leaves of Olea europaea has been dried at a temperature below 35 ° C.

Two. Procedure for obtaining Olea europaea extracts according to claim 1 wherein the extraction is performed with an alkanol with a purity greater than 95% to a temperature below 20 ° C.

Three. Procedure for obtaining Olea europaea extracts according to claims 1 and 2 where the extractant is methanol or ethanol.

April. Procedure for obtaining Olea europaea extracts according to claims 1, 2 and 3 where the extraction is performed three times for 18 hours at a ratio of 1 part per 6 parts leaf solvent.

May. Procedure for obtaining Olea europaea extracts according to claim 1 wherein the purification of the extract is carried out by solvent evaporation under reduced pressure, the concentrated extract solution with purified water and sterilizing filtration per plate.

June. Procedure for obtaining Olea europaea extracts according to claim 1 wherein the evaporation of the solvent is done by lyophilization by freezing at 40 ° C under high vacuum using gentle heat and less than 35 ° C.

July. Procedure for obtaining Olea europaea extracts according to claim 1, 2, 3, 4, 5, 6 where the Oleuropein content greater than 18% and flavonoid content exceeds 5%

August. Use of Olea europaea extracts containing Oleuropein and flanovoides for the manufacture of a medicament for enhancing cellular immunity and delayed hypersensitivity in healthy humans.

9. Use of Olea europaea extracts according to claim 8 wherein the enhancement of cellular immunity and delayed hypersensitivity is characterized by activation and proliferation of T lymphocytes, Natural Killer cells, monocytes and / or granulocytes, as well as increased citoclnas

state of the art.

The first medicinal uses of this extract from the early 1800s, when it was used in liquid form as a treatment for malaria. Since then there have been numerous applications of it have been made in medicine. Among the main effects described for this compound especially noteworthy anti-infective activity against viruses, but also is effective against bacteria, fungi and certain intracellular parasites. The anti-infective activity against pathogens mentioned above has been associated with a direct action thereon elenolic acid, in the case of viruses have been described among other mechanisms product capacity to penetrate the infected cells and directly inactivate viral replication, either by interfering with critical amino acids such as for the virus, or in the case of retroviruses, neutralizing the production of reverse transcriptase or protease. Between the main mechanisms for inactivating the Oleuropein exerts bacteria, highlights a direct lytic action on the outer wall thereof.

No documents within the state of the art has shown the action of the extracts of Olea europaea on the immune system enhancing Cellular Immunity and Delayed Hypersensitivity in humans by activation and proliferation of T infocitos, Natural Killer cells (NK), monocytes and granulocytes in healthy humans.

Membrane antigen CD16, also known as type III receptor for the Fc fragment of IgG, is usually expressed in NK cells into granulocytes and macrophages. Most of the antibody-dependent cytotoxicity or ADCC (Antibody-Dependent Cellular Cytotoxicity) is performed by NK cells and a small population of cytotoxic T lymphocytes also express the marker CD16, the receptor for fixing mainly Fe complex IgG1 and human IgG3. ADCC these cytotoxic cells provides a mechanism by which, taking advantage of the specificity of antigen-antibody binding, may direct or focus their cytotoxic activity. Similarly, the CD16 present in the membrane of granulocytes and macrophages, helps these cells to perform phagocytosis. Thus any agent that increases the expression of this marker in different cell populations, may result in activation of NK cells and a small population of cytotoxic T lymphocytes, which thus will increase in their function as cytotoxic cells. The role that NK cells exert within the immune system is to defend the body against viral infections, eliminating those cells that have been infected by viruses. Increased expression of CD16 on macrophages and granulocytes can enhance the main function of these cells in the inflammatory process, which is the elimination of bacteria, fungi and other pathogens by phagocytosis mechanisms.

On the other hand, for cells of the immune system can perform its function effectively require contacts with other cells or with the extracellular matrix. In this way recognize the state of their environment. Leukocytes therefore not only possess specific receptors on their surfaces that allow them to interact and specifically activated in response to certain stimuli, but also express a wide range of molecules that are included under the denomination of adhesion molecules. These molecules will act as leukocyte ligand receptors to be located in other cells (in this case acting as counter-receptors) or amino acid sequences present in various extracellular matrix proteins such as collagen, fibronectin, laminin and other.

Adhesion molecules, also work in cellular activation coactivator sending signals to the cell interior. According to its structure can be classified into three broad categories: 1. - The selectins 2. - Those that belong to the integrin family.

Three. - Those that belong to the immunoglobulin superfamily. CD1 differentiation antigens CD1 1a and 1b, respectively constitute the alpha chains of integrins LFA-1 and Mac-1, belonging both to the β2 integrin family. These membrane molecules are essential for the normal development of the inflammatory process, primarily mediate the final adhesion of leukocytes to vascular endothelium, extravasation and migration to inflammatory foci. Increasing their expression or de novo expression in the cell membrane, usually as a consequence induces the release of proinflammatory cytokines such as IL-1, TNF-, IL-8 and so on. Inflammation, under normal physiological phenomenon is aimed at eliminating pathogens by phagocytosis and to restore damaged tissue. Increased expression can exercise any drug lymphocytes ay CD11 molecule on lymphocytes and monocytes on the molecule CD11b, along with the increase in plasma levels of proinflammatory cytokines such as IL-1 β and IL-8, activation involves lymphocytes, monocytes and granulocytes to be ultimately translate into an enhancement of the inflammatory response. IL-8, belonging to the chemokine family, also possesses the ability to stimulate the movement of leukocytes (chemokinesis) and directed movement (chemotaxis), especially of neutrophils.

Similarly, increasing the expression of membrane markers CD25 and CD69 in monocytes lymphocytes and monocytes produced by any drug denotes an activation of these populations; potenciándose the immune system with the administration of this drug.

Extraction procedures of Olea europaea described in state of the art provides extracts with a high Oleuropein content, but these documents do not describe the performance Oleuropein on the starting material, ie Olea europaea leaves. The low temperature drying of the leaves of Olea europaea produces a higher yield in the subsequent extraction of the leaves, yielding an extract with a high Oleuropein content without losing the active components maintaining all the pharmacological activity of the extract. Thus, the drying of the leaves of Olea europaea dried at a temperature below 35 ° C provides a starting material having a content of 5% compared to 0.3% Oleuropein described in Prior Art. The use of water for obtaining Olea extracts and subsequent membrane filtration has the advantage of a greater selectivity in the extraction eliminating extract less polar components such as chlorophylls, polyphenols, fats and alkylphenols without pharmacological activity with greater selectivity in the extraction.

proInflamatorias.

10. Use of Olea europaea extracts according to claims 8 and 9 wherein the activation and proliferation of T lymphocytes, Natural Killer cells, monocytes and / or granulocytes are characterized by increased CD16 expression cells, CD11 b, CD11 a, CD14 , CD69 and / or CD25.

11. Use of Olea europaea extract according to claim 8 and 9 wherein the enhancement of cellular immunity and delayed hypersensitivity is characterized by the increase of the cytokines IL-1 β and IL-8.

Oieuropeina action and the flavonoids present in Olea europaea extracts on the immune system by activation of T lymphocytes, Natural Killer cells, monocytes and granulocytes power virucidal and antibacterial action. This is done regardless of viral replication or lysis of the bacterial wall. Furthermore, power cellular immunity against bacteria, viruses and other parasites cell growth and delayed hypersensitivity, favoring the release of proinflammatory cytokines.

OBJECT OF THE INVENTION

The present invention describes a new procedure for obtaining Olea europaea extracts, containing Oleuropein and flavonoids comprising drying processes protected from light at a temperature lower than 35 ° C, extraction by maceration with an alkanol of low molecular weight a temperature below 20 ° C, evaporation of the solvent, treating the extract with water, through membrane filtration and lyophilization.

The present invention discloses a pharmacological activity of the extracts from Olea europaea immune agent, enhancing the antibody-dependent cytotoxicity (ADCC) and more broadly cellular immunity and delayed hypersensitivity by activation and proliferation of T lymphocytes, Natural Killer cells , monocytes and granulocytes in healthy humans in healthy humans.

DETAILED DESCRIPTION OF THE INVENTION

The water content of the leaves of Olea europaea is about 6% as determined by loss on drying for 3 hours at 105 ° C to preserve leaves early dehydration performed at a temperature below 35 ° C protected from light avoiding the decomposition of the active ingredients. It has been found that the leaves of Olea europaea are very sensitive to temperature and light action directly altering their content in active principles.

Content variations on Oleuropein with temperature are detailed in Table 1. Table 1. Oleuropein content under different drying conditions

Secado a 50º C	Secado a 35º C	Secado a T ª Ambiente
1.3%	5.8%	7.3%
1.8%	9.5%	10.1%
4.6%	8.3%	10.1%
3.8%	4.7%	6.6%
2.4%	4.0%	5.8%
3.1%	5.3%	7.6%
3.0%	3.5%	6.8%
1.8%	3.6%	6.5%

So that the drying conditions are critical for the kinetics of degradation of Oleuropein, poor drying conditions cause decomposition of Oleuropein with consequent loss of yield.

The stability has been affected Oleuropein also sunlight, so for Oleuropein content of 3.5% of the dry leaf after sun exposure 12 to 20 ° C passes Oleuropein content to 0.82%. Thus, the drying effect of Olea europaea leaves at a temperature below 35 ° C provides a starting material containing a minimum of 5% compared to 0.3% described state of the art.

Extraction of the leaves is carried out at a temperature below 20 ° C by low molecular weight alkanols with a purity exceeding 95%, the methanol and ethanol alkanols provide better results.

The leaves are extracted with an approximate ratio of leaf per 6 L of solvent by maceration for 18 hours at a temperature below 20 ° C. A liquid extracts are concentrated to obtain a syrupy syrup, preferably under high vacuum at a temperature below 40 ° C until a solids content of around 80%.

Syrupy syrup is treated with purified water and filtered through sterile plates, preventing microbial contamination and pharmacologically inactive components removed from the extract such as chlorophylls, polyphenols, alkylphenols, etc. mucilage.

The solvent is removed from the solution by lyophilization by freezing at -40 ° C under vacuum and mild heat to a temperature below 35 ° C, giving a dry powder green.

Extracts yield is around 25% compared to the starting material and the extract containing more than 18% Oleuropein and 5% flavonoids, being identified by liquid chromatography hesperidin, rutin and luteolin -7 - glucoside extract Olea can be purified by techniques known to one skilled in the art such as chromatography, fractional crystallization, etc.. extracts obtained with a higher content of Oleuropein.

Olea europaea extract has shown pharmacological activity in the immune system in healthy humans after administration of a pharmaceutical composition containing 300 mg of Olea europaea extract, with 18% Oleuropein and 5% flavonoids, together with excipients pharmaceutically acceptable. Receiving doses of study subjects were 300 mg of extract (1 tablet) / 6 hours and 600 mg of extract (2 tablets) / 6 hours. Six individuals thus ingested 1.2 grams of extract and six ingested 2.4 grams of extract.

From EDTA whole blood were analyzed by flow cytometry (Vantage Cell Sorter) and direct immunofluorescence with double labeling (fluorescein-FITC or phycoerythrin-PE), the following parameters:

- Cell line markers (leukocyte populations): CD3, CD4, CD8, CD16, CD19, CD56, CD11a, CD11b and CD14.

Cell activation markers: CD69 and CD25. Combinations of the above monoclonal.

Different gates were selected for analysis, according to the combinations of monoclonal antibodies: lymphocyte analysis gate, for monocytes and polymorphonuclear cells. Monoclonal combinations were:

- CD3-FITC/CD25-PE

- CD4-FITC/CD8-PE

- CD19-FITC/CD25-PE - CD3-PE / CD16-FITC/CD56-FITC

- CD16-FITC/CD56-FITC/CD25-PE - CD11a-FITC/CD25-PE - CD11b-FITC/CD25-PE - CD14-FITC/CD25-PE

All these parameters were analyzed before taking the drug (DO) at 24 hours after taking the drug (D1), at 72 hours (D3) and at day 11 (D11). Therefore performed a total of 4 extractions (10 mL / extraction) to each patient. Besides EDTA blood tube, two tubes were used for serum in each of the four extractions, one of which was used for elemental analysis and other biochemical frozen at -20 ° C until further use. The latter tube was used for the determination of plasma levels of cytokines by ELISA. Cytokines measured were interleukin-1 β (IL-1β), interleukin 2 (IL-2), interleukin 8 (IL-8) and interferon gamma (INF-γ). The results of the study were: 1. - No significant changes in the basic biochemistry or hematology analysis before and after consuming the product.

Two. - No significant difference between the group that took 1.2 gr. / Day and the group that took 2.4 gr. / Day.

Three. - There is a tendency to increase the percentage of cells with CD16 expression, both in lymphocytes and granulocytes. April. - There is an increase in the percentage of cells with expression of CD11 b, both in lymphocytes and in monocytes and increased the expression of CD11 on monocytes and not appreciate significant changes in expression kinetics of lymphocyte populations CD3 +, CD4 + , CD8 + and CD19 +. May. - There is an increase in the expression of CD14 on monocytes. June. - Are observed significant and important increases in the expression of the early activation markers (CD69) and late (CD25), both in lymphocytes and in monocytes. July. - Tendency to increase plasma levels of certain proinflammatory cytokines such as IL-1 β and IL-8, maximum on the third day of taking the product.

From the above results show the enhancement of cellular immunity and delayed hypersensitivity in the activation of T lymphocytes, NK, monocytes and granulocytes, as well as by increased plasma levels of inflammatory cytokines.

The invention is described by way of examples, not being limiting to the scope thereof.

Example 1. Illustrates the extraction of the leaves of Olea europaea.

106 Kilograms of Olea europaea leaves with a water content of 6%, are dried in a forced air artificial drying by convection at a temperature below 35 ° C.

The dried leaves are crushed by a cryogenic process. Dried leaves of Olea europaea with a Oleuropein content of 6.23% are extracted with 600 liters of 95% methanol for 18 hours at 20 ° C. The process is repeated 3 more times until extraction exhaust. Filtered liquid extracts are concentrated under vacuum at less than 40 ° C to obtain 32 kg of syrup syrupy. The syrup was suspended in water and purified by sterile filtration. The aqueous solution was concentrated by lyophilization to yield 25 kg dry extract of Olea europaea, with Oleuropein content of 21.9% and 5% in flavonoids (hesperidin, rutin, luteolin-7-glucoside). Extract yield 25%. Ejermplo 2. Illustrates the variation of the immunological parameters in healthy humans with intake Olea extract.

Parámetro	T=0	1 dia	3 dias	10 dias
LinfocitosCD16+ (%)	15	20	21	24
LinfocitosCD11a+(%)	7	11	27	27
MonocitosCD11b+ (%)	72	71	83	92
MonocitosC14+(%)	69	67	81	84
CD69(%)	3	3	14	10
CD25 (%)	1	5	16	12
IL -1ß (pg/ml)	6	32	30	4
IL-8(pg/ml)	101	180	181	169

Example 3. Illustrates the pharmaceutical formulation used.

Olea europaea Extract 300 mg

154 mg microcrystalline cellulose

Magnesium stearate 3 mg

PATENT CITATIONS

Cited Patent	Filing date	Publication date	Applicant	Title
WO1996014064A1 *	Nov 3, 1995	May 17, 1996	William R Fredrickson	Method and composition for antiviral therapy
WO1999038383A1 *	Feb 2, 1998	Aug 5, 1999	Nachman Leslie	Method for producing extract of olive leaves and extract produced thereby
FR2507477A1 *				*Title not available*

* Cited by examiner

NON-PATENT CITATIONS

Reference		
1	*	DE PABLO M.A. ET AL.: 'Influence of diets containing olive oil, sunflower oil or hydrogenated coconut oil on the immune response of mice' J. CLIN. BIOCHEM. NUTR. vol. 25, no. 1, 1998, pages 11 - 23, ISSN 0912-0009, XP002947756
2	*	SANDERSON P. ET AL.: 'Effects of dietary lipid manipulation upon rat spleen lymphocyte functions and the expression of lymphocyte surface molecules' J. NUTR. ENVIRON. MED. vol. 5, no. 2, 1995, pages 119 - 132, ISSN 1359-0847, XP002947758
3	*	See also references of EP1157701A1
4	*	YAQOOB P. ET AL.: 'Inhibition of natural killer cell activity by dietary lipids' IMMUNOLOGY LETTERS vol. 41, no. 2-3, 1994, pages 241 - 247, ISSN 0165-2478, XP002947757

* Cited by examiner

CLASSIFICATIONS

International Classification	A61P43/00, A61P37/04, A61K36/18, A61P37/02, A61K36/00, A61K36/63
Cooperative Classification	A61K36/63, Y10S514/885
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LEGAL EVENTS

Date	Code	Event	Description
Jul 1, 2005	WWW		**Ref document number:** 2000985273 **Country of ref document:** EP
Mar 8, 2002	WWE		**Ref document number:** 09913029 **Country of ref document:** US
Nov 28, 2001	WWP		**Ref document number:** 2000985273 **Country of ref document:** EP
Sep 21, 2001	WWE		**Ref document number:** 2000985273 **Country of ref document:** EP
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Aug 21, 2001	ENP		**Ref country code:** JP **Ref document number:** 2001 548129 **Kind code of ref document:** A **Format of ref document f/p:** F
Aug 16, 2001	WWE		**Ref document number:** 21740/01 **Country of ref document:** AU
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